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Abstract

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Biochemistry | Biophysics | Structural Biology

Comments

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Title: **Probing enzymatic structure-function in the di-hydroxylating sesquiterpene synthase ZmEDS**

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Running title: The catalytic mechanism of ZmEDS

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Key words: ZmEDS, hedycaryol, terpene synthase, site-directed mutagenesis, catalytic mechanism

Abstract

Terpene synthases(TPSs) play a vital role in forming the complex hydrocarbon backbones that underlie terpenoid diversity. Notably, some TPSs can add water prior to terminating the catalyzed reaction, leading to hydroxyl groups, which are critical for biological activity. A particularly intriguing example of this is the maize (*Zea mays*) sesquiterpene TPS whose major product is eudesmanediol, ZmEDS. This production of dual hydroxyl groups is presumably enabled by protonation of the singly-hydroxylated transient stable intermediate hedycaryol. To probe the enzymatic structure-function relationships underlying this unusual reaction, protein modeling and docking were used to direct mutagenesis of ZmEDS. Previously, a F303A mutant was shown to produce only hedycaryol, suggesting a role in protonation. Here this is shown to be dependent on steric bulk positioning of hedycaryol, including a supporting role played by the nearby F299, rather than π-cation interaction. Among the additional residues investigated here, G411 at the conserved kink in helix G is of particular interest, as substitution for this leads to predominant production of the distinct(-)-valerianol, while substitution for the aliphatic I279 and V306 can lead to significant production of the alternative eudesmane-type diols 2,3-*epi*-cryptomeridiol and 3-*epi*-cryptomeridol, respectively. Altogether, nine residues important for this unusual reaction were investigated here, with the results not only emphasizing the importance of reactant positioning suggested by the stereospecificity observed between the various product types, but also highlighting the potential role of the Mg^{2+}-diphosphate complex as the general acid for the protonation-initiated (bi)cyclization of hedycaryol.

Introduction

Terpene synthases (TPSs) catalyze complex reactions that transform acyclic precursors into often intricately cyclized and/or rearranged hydrocarbon structures that provide the diverse skeletal backbones underlying the astounding structural variation observed in the terpenoids, which represent the largest class of natural products[1]. Their catalytic mechanism relies on heterolysis of the allylic diphosphate ester bond in their substrate to initiate carbocationic cascades that cyclize and/or rearrange these relatively simple isoprenyl precursors. The reaction is then terminated by deprotonation, generally directly of the final carbocation, but in certain cases following the addition of water. This leads to either the typical production of a hydrocarbon or more atypical production of (singly) hydroxylated, terpenes, respectively.

Notably, terpenoid natural products often contain at least two spatially separated oxy groups, which both impart solubility as well as hydrogen-bonding capacity to enable specific binding to (macro)molecular targets[2]. Given the hydrophobic nature of hydrocarbons, these oxygens are generally inserted by cytochrome P450 mono-oxygenases, which typically act on such substrates[2]. However, while it has been evident for some time that TPSs can produce singly hydroxylated terpenes[1], it has been recently reported that these can further yield di-hydroxylated products. In particular, two TPSs act on the general sesquiterpene precursor [*E,E*]-farnesyl diphosphate (FPP) and catalyze production of eudesmane-type diols, with that from maize producing eudesmanediol, ZmEDS[3], and that from *Tripterygium wilfordii* producing cryptomeridiol, TwCS[4]. These sesquiterpenoids can then directly exert biological activity – e.g., cryptomeridiol is the principal active component of the plant-derived antispasmodic Proximol.

Eudesmane-type, as well as derived (i.e., rearranged), sesquiterpenes contain adecalin bicycle produced by a particularly unusual mechanism. The reaction involves initial (macro)cyclization of the initiating allylic carbocation via 1,10 ring closure, with subsequent deprotonation forming a stable germacrane-type sesquiterpene. However, this intermediate is then immediately subjected to protonation of the 6,7(endocyclic) olefin, with the resulting carbocation undergoing secondary (bi)cyclization, which can be followed by rearrangement, before terminating deprotonation occurs. While labeling studies clearly indicate formation of such stable intermediates, these do not serve as endogenous substrates. This reaction mechanism was first elucidated in formation of aristolochene, a typical olefinic sesquiterpene TPS product derived via methyl migration from the eudesmanyl carbocation formed by (bi)cyclization prior to direct deprotonation[5]. Intriguingly, it has been proposed that protonation of the corresponding germacrene A intermediate by the tobacco (*Nicotiana tabacum*) 5-*epi*-aristolochene synthase (TEAS) critically depends on a specific tyrosine residue (Y520) whose hydroxyl group protrudes into the top of the active site. In particular, as the TEAS:Y520F mutant has been reported to only generate germacrene A as its sole product[6].

In the case of the eudesmane-type diols, the reaction presumably proceeds via formation of the germacrane-type hydroxylated derivative hedycaryol (see Scheme 1). Indeed, hedycaryol was clearly identified as a minor component of the products with ZmEDS and labeling studies demonstrated protonation of the reactant[3]. However, while the key tyrosine identified in TEAS is conserved in ZmEDS (indeed this residue is very widely conserved in TPSs), and phenylalanine substitution for this (Y529F) significantly increased the proportion of hedycaryol, eudesmane-type sesquiterpene products were still readily evident. By contrast, alanine substitution for a phenylalanine residue suggested to line the bottom of the active site by protein modeling (F303A) led to essentially exclusive production of hedycaryol. Here further mutagenesis of F303 was carried out to demonstrate that this residue is important for positioning rather than activation of hedycaryol for protonation, along with investigation of additional residues identified by protein modeling and docking studies, to clarify the enzymatic structure-function relationships underlying the highly unusual reaction catalyzed by this di-hydroxylating sesquiterpene synthase.

Methods

Sited-directed mutagenesis and recombinant expression—Site-directed mutagenesis of ZmEDS was accomplished using the Strategene Quikchange kit following the manufacturer's instruction with the previously reported pET28a derived construct[3]. The primers used for mutagenesis are listed in Table S1. The resulting ZmEDS mutant expression vectors were co-transformed into *Escherichia coli* BL21 (DE3) competent cells with pMevT-MBIS, which increases metabolic flux to FPP, as described previously[3, 7]. These recombinant strains were grown in 5 mL NZY liquid medium at 37°C with 200rpm shaking overnight. The culture was transferred into 50 mL medium and allowed to grow until the OD_{600} was 0.8~1. After induction with 1mM IPTG, the cultures were grown at 16 °C overnight and then extracted twice with an equal volume of hexanes. The organic extract was separated and concentrated via rotary evaporation for GC-MS analysis, which was carried out as described previously[8].

Sequence alignment, homology modeling and molecule docking— Sequence alignment was carried out with CLC Sequence Viewer 8 with selected sesquiterpene synthases. Their accession numbers are as follows, ZzES (*Zingiber zerumbet*, β-eudesmol synthase, B1B1U4.1), ChTPS1 (*Camellia hiemalis,* valerianol synthase, BBC44642.1), TwCS (*Tripterygium wilfordii*, AWV55521.1), TEAS (*Nicotiana tabacum*, 5-*epi*-aristolochene synthase, 5EAS_A), ScGAS (*Solidago canadensis*, germacrene A synthase, CAC36896.1), ZmTPS4 (*Z.mays*,7-*epi*-sesquithujene synthase, NP_001292867.2), ZmTPS5 (*Z. mays*, sesquithujene synthase, Q6JD70.1), AaADS (*Artemisia annua*, amorpha-4,11-diene synthase, Q9AR04.2),

and AaGAS (*Artemisia annua*, germacrene A synthase, ABE03980.1)[4, 9-13]. A modeled structure for ZmEDS was generated with the SWISS-MODEL online tool, using TEAS (PDB ID: 5EAS)[14] as the template, as previously reported[3]. The conformation of the small molecules for docking were derived from energy minimization calculations using ChemBio3D Ultra 14.0 and used to generate PDB files for further analysis. Docking was accomplished with AutoDock 4.2.6. The resulting complexes were visualized with PyMOL 1.8.x. A Mg^{2+}-diphosphate complex was placed into ZmEDS by modeling with a distinct TEAS structure(PDB ID: 4RNQ)[15] as the template, and then placing the Mg^{2+}-diphosphate complex from this into the modeled ZmEDS structure, much as previously described for other TPSs[16].

Products purification and identification—To obtain sufficient amount of terpenoid products for chemical structure elucidation, the culture scale was increased to 6 L. Terpenoids were extracted as above and isolated through silica-gelchromatography. The new product, valerianol, produced withZmEDS:G411A, was purified through eluting with the hexane and EtOAc mixture (5:1, v/v) in the first round. After dried with nitrogen gas, it was eluted by a mixture of hexane and dichloromethane (2:1, v/v) in the second round. Another product, eremophil-1-en-11-ol was produced by the mutantZmEDS:V306A. The compound was first eluted by a mixture of hexane and EtOAc (15:1, v/v), then purified with elution of the mixture of hexane and dichloromethane (1:1, v/v) after dried and loaded on a new silica column.

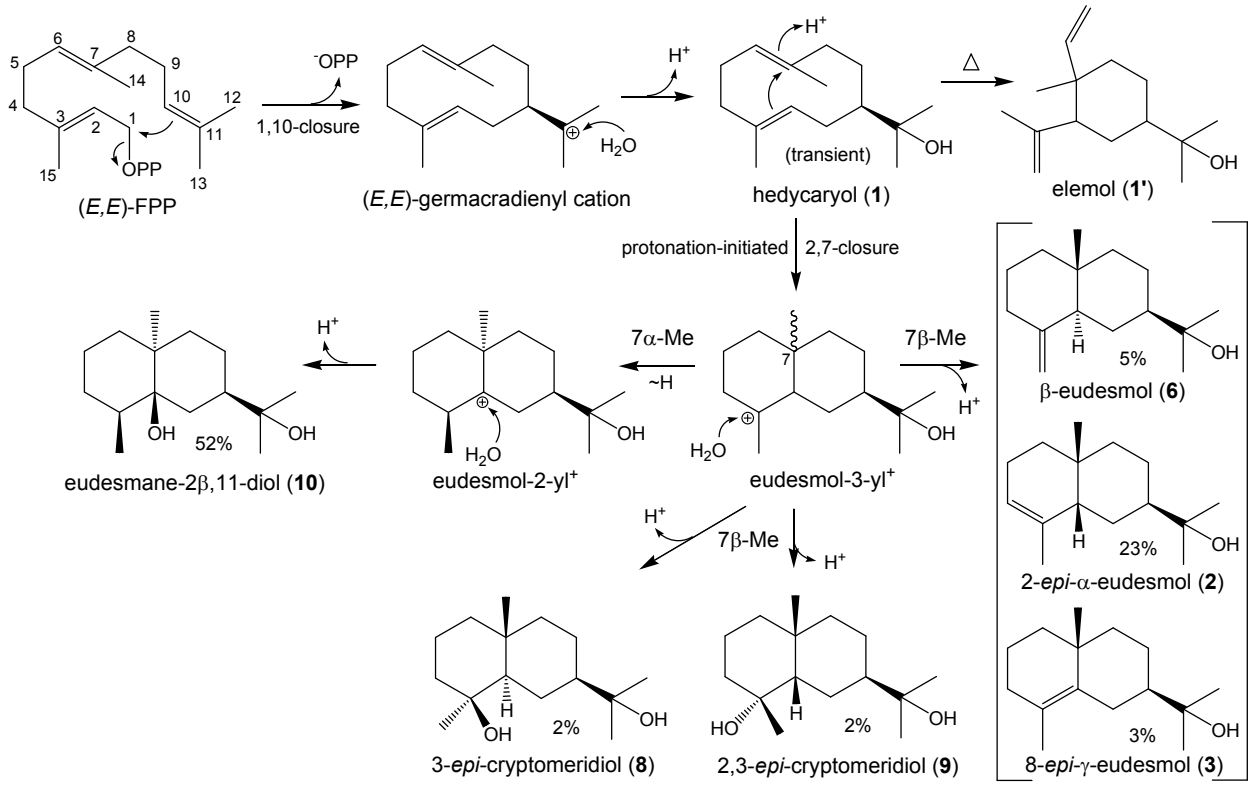
NMR analysis—The purified terpenoid products were dissolved in 0.5 ml $CDCl_3$ and transferred into a 2.5 mm × 100 mm NMR tube for NMR analysis, which was carried out on a Bruker Avance 600 MHz instrument with ^{1}H and ^{13}C spectroscopic analysis at 22°C using a 5-mm TXI CryoProbe. Comparison of the NMR chemical shift data (TableS2)to previously reported literature values identified these as valerianol and eremophil-1-en-11-ol[17, 18].

Results and Discussion

Reactant configuration largely dictates product outcome

While the product profile of ZmEDS has been previously reported[3], careful analysis reveals the key role played by reactant configuration. This is primarily observed from the major effect exerted by the 7-epimeric configurations of the eudesmol-3-yl carbocation intermediate formed by protonation-initiated (bi)cyclization of the transient stable intermediate hedycaryol (Scheme 1). In particular, if the 2,7-ring closure occurs via addition to the *re* face of the 6,7-olefin, that generates the 7α-methyl (Me) epimer of eudesmol-3-yl^{+}. This then undergoes 1,2-hydride transfer (from carbon-2, C2, to C3), generating the depicted stereoisomer of eudesmol-2-yl^{+}, to which the (second) water is added prior to deprotonation, forming the primary product eudesmanediol (~52%, see Table S3for product ratios of wild-type and all

mutants of ZmEDS reported here). However, if (bi)cyclization occurs via addition to the *si* face of the 6,7-olefin, that generates the 7β-Me epimer of eudesmol-3-yl$^+$. This then generally undergoes direct deprotonation to generate a eudesmol derived olefin, although addition of water can also occur, generating the alternative diol products 3-*epi*-cryptomeridiol and 2,3-*epi*-cryptomeridiol.Accordingly, the configuration at C7 in the eudesmol-3-yl$^+$ formed by protonation-initiated (bi)cyclization dictates the further course of the catalyzed reaction, emphasizing the importance of reactant configuration on product outcome.



Scheme 1. Effect of C7 stereochemistry on ZmEDS product outcome.

Selecting residues for investigation

To screen for residues that might contribute to the unusual activity of ZmEDS, a combination of sequence alignment, homology modeling and molecule-protein docking was carried out. A modeled structure of ZmEDS based on the previously determined crystal structure of TEAS[14], into which the FPP analog and divalent magnesium (Mg^{2+}) ions co-crystallized with TEAS can be easily placed, has previously been reported[3]. Here the presumed intermediate hedycaryol and the major product eudesmanediol were docked into the ZmEDS model (Figure 1).To provide additional insight into residues that might contribute to its

unusual activity, ZmEDS was aligned with a number of other functionally characterized TPSs that produce eudesmane-type sesquiterpenes, particularly including the 'other' diol producing TwCS, as well as others that yield (singly) hydroxylated products (Figure 2). Based on active site location, particularly the feasible interactions with the substrate (FPP), intermediate (hedycaryol) and/or product (eudesmanediol), as well as the lack of conservation in other TPSs, ZmEDS residues I279, F299, F303, V306, Y384, T409, S410, G411 and N453 were selected for further exploration.

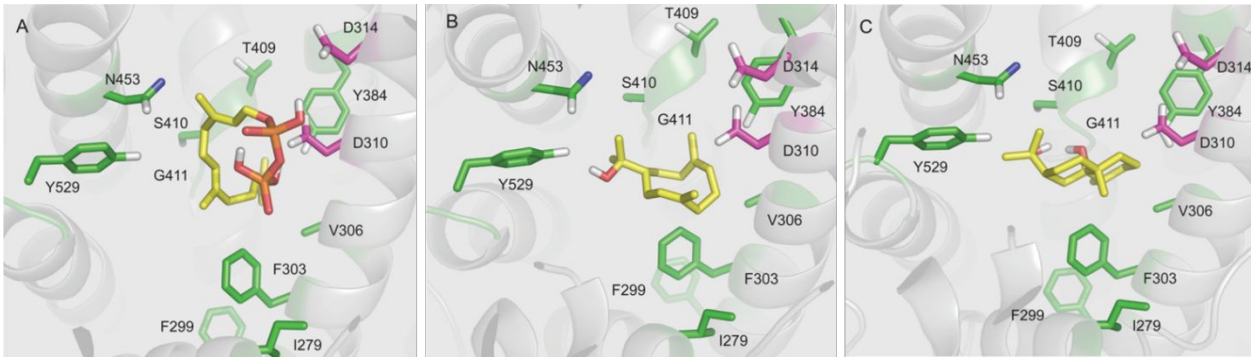


Figure 1. Docking into the ZmEDS model. Shown is the catalytic site with side chains of key residues depicted in stick format for docked substrate (FPP) (**A**), reactant (hedycaryol) (**B**) or major product (eudesmanediol) (**C**). The docked molecules are shown with yellow carbon skeletons and orange pyrophosphate group or hydroxyl groups in red and gray. The first and last aspartate residues in the D_{310}DxxD motif are shown in purple (carbon skeletons) and gray (carboxyl groups). The residues investigated here are indicated in green with gray hydroxyl groups or the amide group in blue and white.

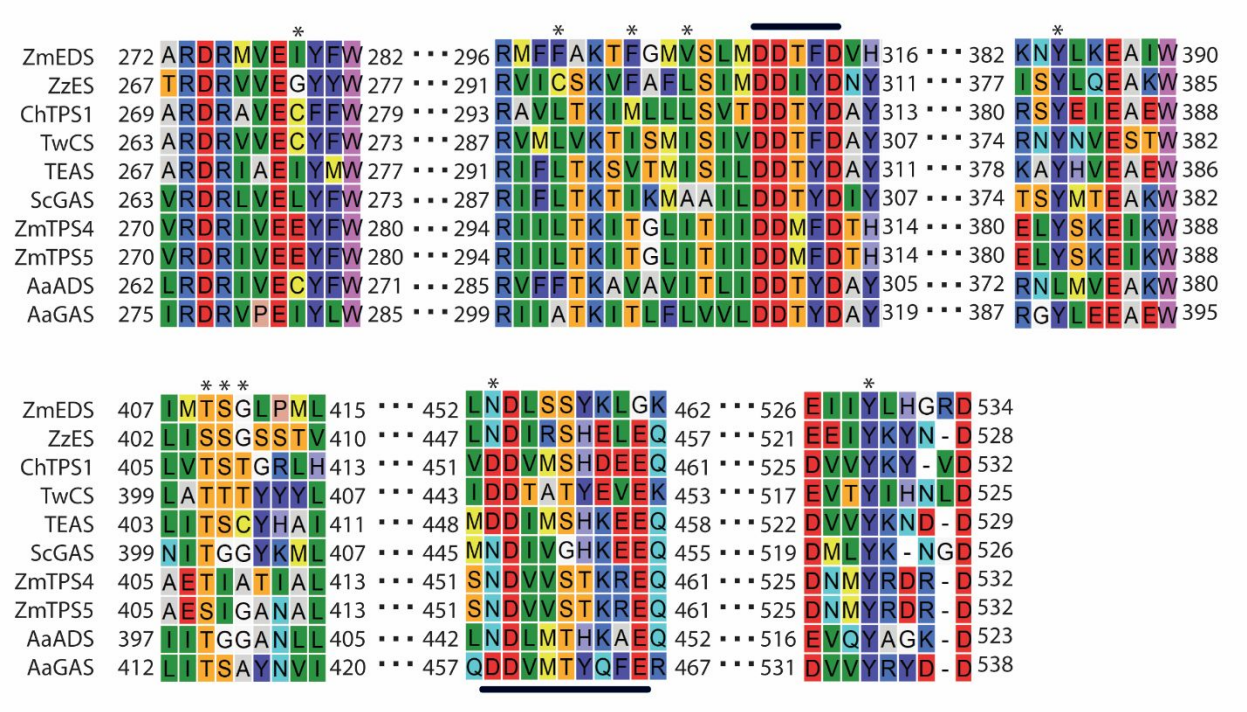


Figure 2. Alignment of selected plant sesquiterpene synthases. The residues investigated in this study are labeled with asterisks. The two conserved magnesium binding motifs DDxxD and (N,D)Dxx(S,T)xxxE are indicated by bold lines above or below the alignment, respectively.

Steric bulk effects on reactant positioning

Previous work indicated that F303 was important for protonation of the transient stable intermediate hedycaryol, as ZmEDS:F303A produces only hedycaryol[3]. The importance of F303 might arise from either quadrapole stabilization of the incipient carbocation resulting from protonation, or steric bulk positioning of the reactant. Further mutagenesis strongly indicates the latter, as ZmEDS:F303L primarily produces a mixture of α- and β-eudesmol, both derived from protonation initiated (bi)cyclization of hedycaryol (Figure 3A). Notably, the positioning of F303 is assisted by an additional phenylalanine which lies underneath (Figure 1). Mutagenesis of this F299 further supports the importance of this 'lower' region of the active site for reactant position by steric bulk, as ZmEDS:F299L also primarily produces a mixtureof α- and β-eudesmol (Figure 3B). These result via direct deprotonation from alternative neighboring carbons of the eudesmol-3-yl$^+$ formed by protonation-initiated (bi)cyclization of hedycaryol (Scheme 1), but very little diol products are evident, so this positioning effect also is important for such addition of the(second) water.

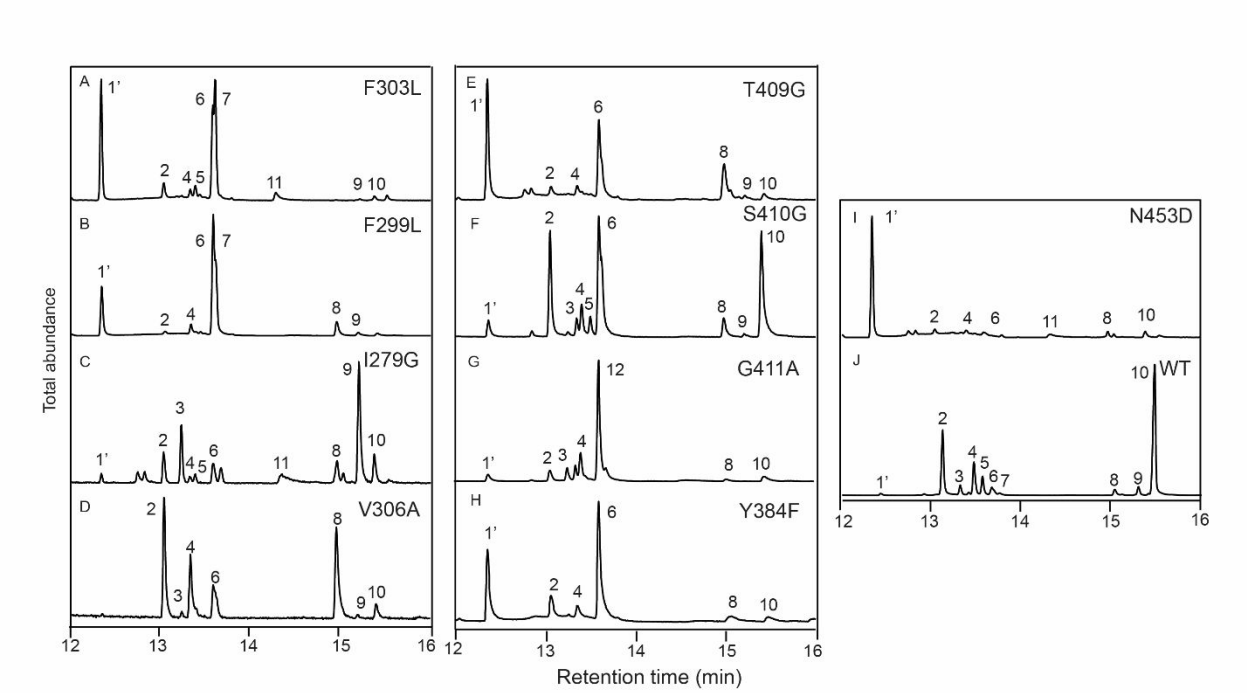


Figure 3. GC-MS analysis of ZmEDS mutants. Chromatograms are shown for products of ZmEDS mutants (**A** to **I**), as well as wild-type (WT) ZmEDS for comparison (**J**). All peaks are numbered consistently throughout: **1'**,elemol (the thermally rearranged product from hedycaryol (**1**), see Scheme 1); **2**, 2-*epi*-α-eudesmol; **3**, 7-*epi*-γ-eudesmol; **4**,eremophil-1-en-11-ol; **5**,unidentified; **6**,β-eudesmol; **7**, α-eudesmol; **8**,2,3-*epi*-cryptomeridiol; **9**, 3-*epi*-cryptomeridiol; **10**, eudesmane-2α,11-diol; **11**,farnesol; **12**,(-)-valerianol.

The two aliphatic residues I279 and V306 are located on the 'side' of the active site near F303 in the ZmEDS model (Figure 1). Given the hypothesized importance of steric bulk effects for reactant positioning and product outcome, both of these residues were mutated to amino acids with smaller side chains. Interestingly, rather than affecting protonation of hedycaryol, these mutations led to alterations in the ratio of eudesmane-type diol products. Instead of eudesmanediol, ZmEDS:I279G primarily generated3-*epi*-cryptomeridiol (Figure 3C), while ZmEDS:V306Agenerated 2,3-*epi*-cryptomeridiol as its main diol product (Figure 3D). In contrast to the production of eudesmanediol, which requires a preceding 1,2-hydride shift, the cryptomeridiols result from addition of water to the eudesmolyl carbocation directly formed by the protonation-initiated (bi)cyclization of hedycaryol. Accordingly, I279 and V306 are important for this 1,2-hydride shift, as well as affecting which face of the carbocation the (second) water is added to, leading to these 2-hydroxy epimers of 3-*epi*-cryptomeridiol. Given the inert nature of these aliphatic residues, their effects presumably result from altering the positioning of the

reactant in the active site, particularly of the eudesmolyl^{+} intermediate relative to the second water molecule to be added.

In addition, it should be noted that ZmEDS:V306A generated a sufficiently high enough proportion of a previously unidentified product (corresponding to peak 4) to enable its purification and subsequent identification as eremophil-1(10)-en-11-ol by NMR analysis (Figure 4A, Table S2). Formation of this requires not only the 1,2-hydride shift also necessary for generation of eudesmanediol, but also a1,2-methyl shift to form the eremophilol backbone, as well as subsequent 1,4-hydride shift, which further highlights the effect these residues have on positioning of the ensuing reactant relative to the second water molecule to be added rather than the preceding protonation-initiated (bi)cyclization of hedycaryol (Scheme S1).

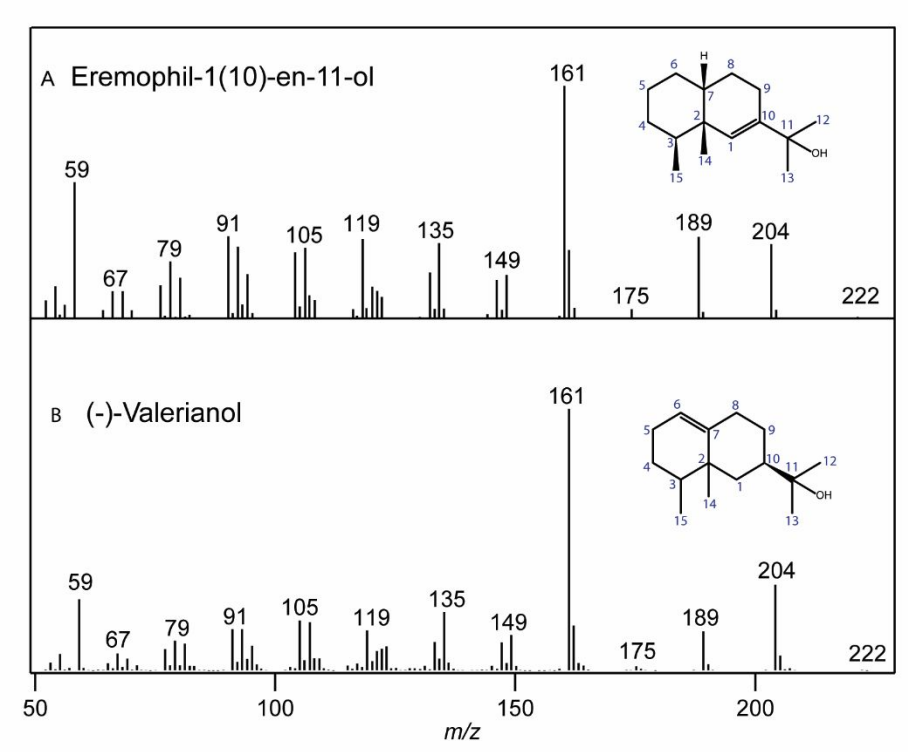


Figure 4. Mass spectra and chemical structures of eremophil-1(10)-en-11-ol and (-)-valerianol.

The importance ofthe G1/2 helix-break

The G1/2helix-breakis conserved in TPSs, but the amino acid residues that make up this kink are diverse[1], and changes to these residues have been shown to have profound effects on product outcome[16, 19]. The ZmEDS model indicates that the corresponding residues are $T_{409}S_{410}G_{411}$ (Figure 1). A number of mutants were made for each of these residues. Generally, substitution with larger residues led to decreased

enzymatic activity. In addition, the conservative mutations T409S and S410T did not significantly change the observed product profile (Table S3). By contrast, substitution of glycine for either T409 or S410 led to significant and distinct effects on the product profile. WithZmEDS:T409G there was a significant decrease in diol products, with corresponding increase in hedycaryol, as well as α- and β-eudesmol (Figure 3E), while ZmEDS:S410G only generated higher amounts of α- and β-eudesmol (Figure 3F).This suggests thatT409 is more important than S410 for generation of eudesmanediol.

Although G411 is already the minimal amino acid side chain, substitution with the next smallest alanine not only left ZmEDS catalytic activity intact, but also strikingly led to generation of a new product (Figure 3G). In particular, ZmEDS:G411A generated almost no diols, instead predominantly producing a new mono-hydroxylated sesquiterpene. This was generated in sufficiently high enough amounts to enable purification and subsequent identification as valerianol by NMR analysis (Figure 4B, Table S2). Production of valerianol requires a 1,2-methyl shift in the eudesmolyl$^+$ intermediate prior to direct deprotonation (Scheme S2), indicating that G411 is important for preventing such further rearrangement.

While it has been suggested that the G1/2 helix-break is important for the initial 1,10-ring closure[20], these mutants do not seem to affect this first (macro)cyclization, but instead the subsequent protonation-initiated (bi)cyclization and/or rearrangement, as well as second addition of water. Although the ability of hydroxylated amino acid side-chains to serve as catalytic bases deprotonating carbocations in TPSs has been shown[16], the effect of the T409 and S410 mutants do not seem to indicate such a role for these residues in ZmEDS either. On the other hand, it could be argued that T409 might play a role in positioning the water to be added to the eudesmolyl carbocation. Nevertheless, combined with the effect of the G411A mutation, it appears that these residues at the least play a role in positioning the reactant as well as allowing access to the second water for addition.

A role for active site tyrosines

In contrast to previously reported results with TEAS[6], it has been indicated that Y529 is not absolutely required for protonation of the transient stable intermediate hedycaryol, as ZmEDS:Y529F still produces eudesmane-type sesquiterpenes[3]. In the ZmEDS model, Y384 also has its hydroxyl group protruding into the active site, on the opposite side toY529 (Figure 1). However, mutagenesis indicated that this does not serve as the catalytic acid either, as ZmEDS:Y384F generated β-eudesmol as its major product (Figure 3H). Intriguingly, it has been suggested that the highly conserved tyrosine corresponding to Y529 might be involved in binding the diphosphate moiety of the substrate[21], and Y384 might also serve a similar function (i.e., serve as outer shell ligand). Consistent with this hypothesis, modeling of ZmEDS against a TEAS structure solved in the presence of a cyclized substrate analog with the Mg^{2+}-pyrophosphate co-product also still present[15], suggests that both of these tyrosines are within hydrogen bonding distance of

this complex (Figure 5). Particularly given the previous suggestion that the pyrophosphate co-product may serve as a Brønsted acid/base in many TPSs[22], this hints at a further role for this group in the unusual reaction catalyzed by ZmEDS.

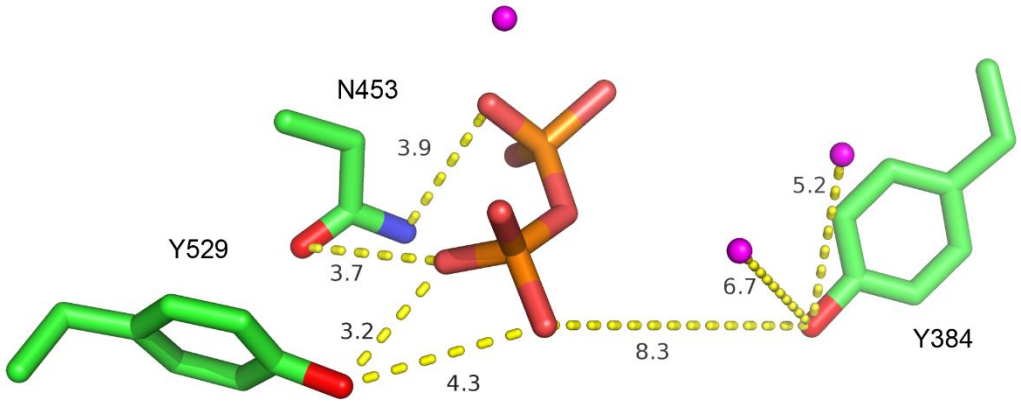
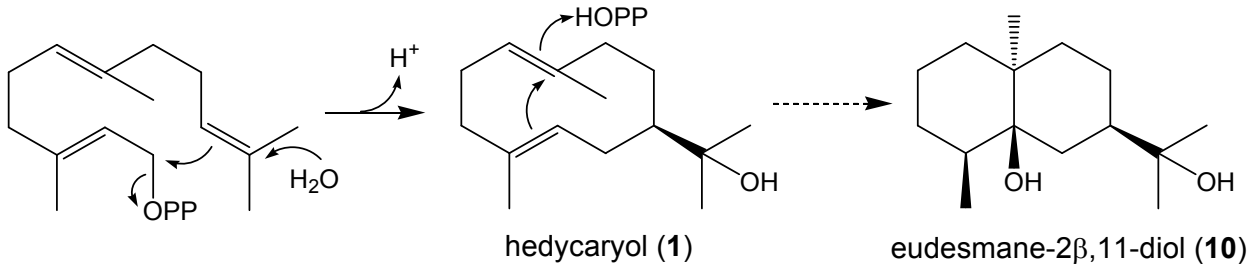


Figure 5. Mg^{2+}-diphosphate complex interactions with targeted residues. The side chains of Y384, N453 and Y529 from the modeled ZmEDS structure are depicted in relation to the Mg^{2+}-diphosphate complex, all in stick format with standard coloring – i.e., green (carbon), red (oxygen), blue (nitrogen), orange (phosphate) and purple (magnesium). The distances of potential hydrogen bonds also are shown.

A role for the magnesium-diphosphate co-product complex

In order to investigate the potential role of the diphosphate co-product, further mutagenesis was carried out. In particular, to bind the diphosphate and enable lysis, TPSs utilize a trio of divalent magnesium cations as co-factors, which are ligated by two highly conserved motifs, DDxxD and (N,D)Dxx(S,T)xxxE[1]. The second motif obviously exhibits some variability, with the identity of the first residue clearly affecting the electrostatic nature of the liganded Mg^{2+}-diphosphate complex, both in the substrate but also anionic co-product[21]. The effect of varying this residue (N453) was investigated here in ZmEDS. Strikingly, ZmEDS:N453D seems to efficiently produce almost exclusively hedycaryol (Figure 3I). This suggests that the liganded Mg^{2+}-diphosphate complex is important for the protonation of hedycaryol required to produce the derived eudesmane-type sesquiterpenes, and hints that this co-product may serve as the relevant catalytic acid (Scheme 2). To the extent that Y529 and/or Y384 are involved in ligating the Mg^{2+}-diphosphate complex, such a role for this would further be consistent with the effect of the Y529F, and possibly also Y384F, mutation(s) on product outcome as well.



Scheme 2. Potential role of pyrophosphate co-product as catalytic acid for protonation of hedycaryol.

Conclusions

Here the enzymatic structure-function relationships underlying the unusual reaction catalyzed by ZmEDS were probed by site-directed mutagenesis. The results not only emphasize the importance of reactant positioning suggested by the stereospecificity of the observed products, but also highlight the potential role of the Mg^{2+}-diphosphate complex as the general acid for the protonation-initiated (bi)cyclization of the transient stable intermediate hedycaryol. In the course of these studies mutants were found that mediate selective product outcome, not only for already known minor diol products (i.e., I279G and V306A), but also a new product (i.e., G411A). The ability of ZmEDS:I279G and ZmEDS:V306A to provide access to substantial amounts of3-*epi*-cryptomeridiol and 2,3-*epi*-cryptomeridiol (respectively), further provides novel biosynthetic access to these sesquiterpene diols. In addition, the evidence reported here supporting use of the Mg^{2+}-diphosphate complex co-product as a general acid provides deeper insight into TPS activity, as such protonation-initiated cyclization of transient stable intermediates is a feature of many of these enzymes[1]. Indeed, the ability to manipulate this aspect of the catalyzed reaction by altering the residues identified here, both the highly conserved tyrosine (Y529) and, especially, first residue in the (N,D)Dxx(S,T)xxxE motif, immediately suggests how such enzymes might be manipulated to both probe mechanism and provide access to the relevant stable intermediates.

Accession IDs

ZmEDS: NP_001148146.

Supporting Information

The Supporting information associated with this manuscript is available free of charge on the ACS website:

Primers used for site-directed mutagenesis (Table S1), ^{13}C NMR chemical shift data of valerianol and eremophil-1(10)-en-11-ol (Table S2), Product distribution of wild-type ZmEDSand mutants (Table S3), Extended reaction from stereospecific eudesmol-2-yl$^+$ to eremophil-1-en-11-ol (Scheme S1), and Alternative reaction from stereospecific eudesmol-2-yl$^+$ to valerianol (Scheme S2).

Author contribution statement

QW conceived the research. JL, LW, QS and JF conducted the experiments and collected all the data. J. Liu analyzed NMR data and elucidated the chemical structures. JL and QW analyzed all the data and wrote the paper with help of RJP.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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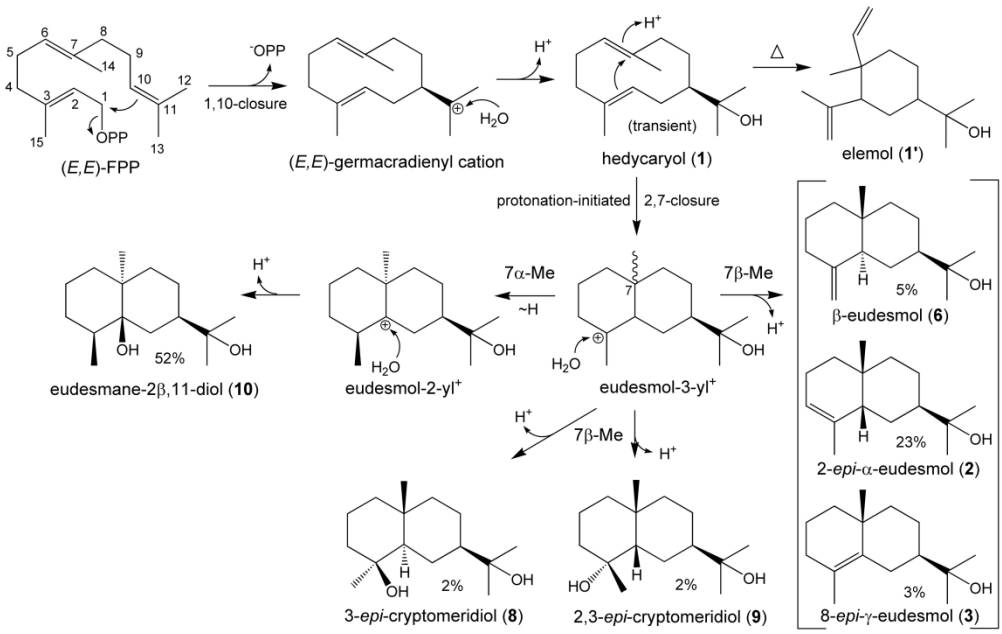

Scheme 1

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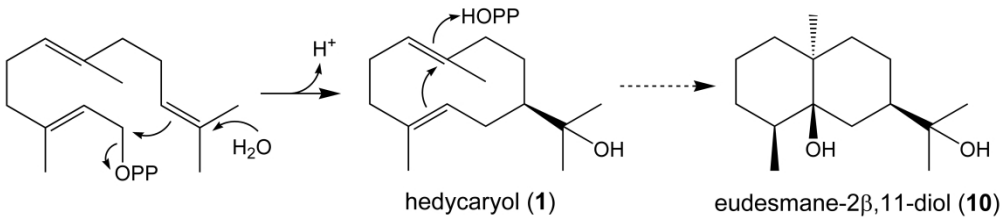
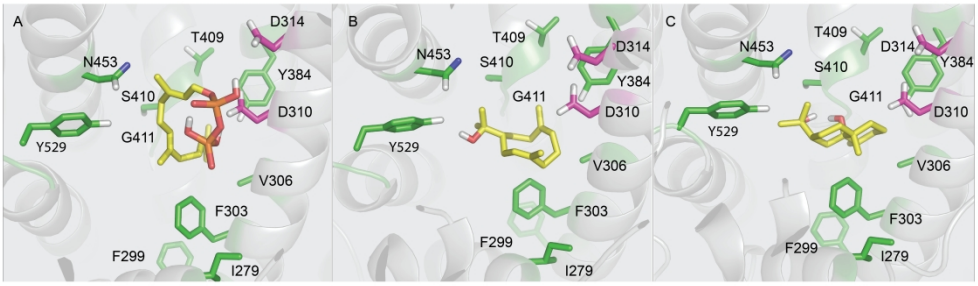


Scheme 2

237x51mm (600 x 600 DPI)



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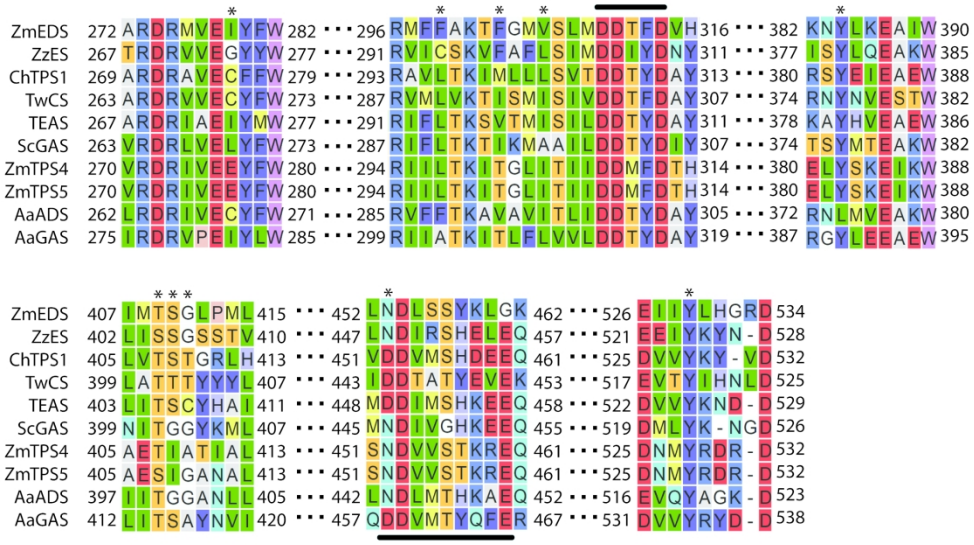

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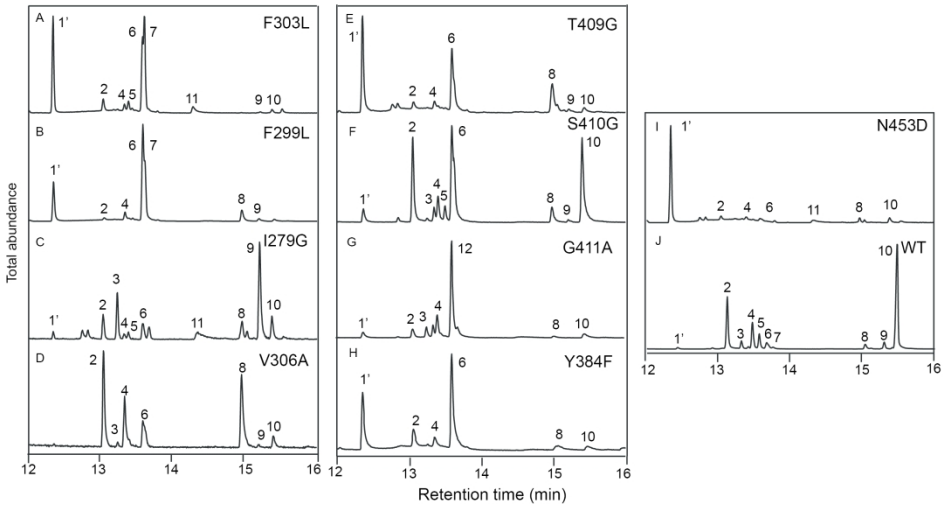

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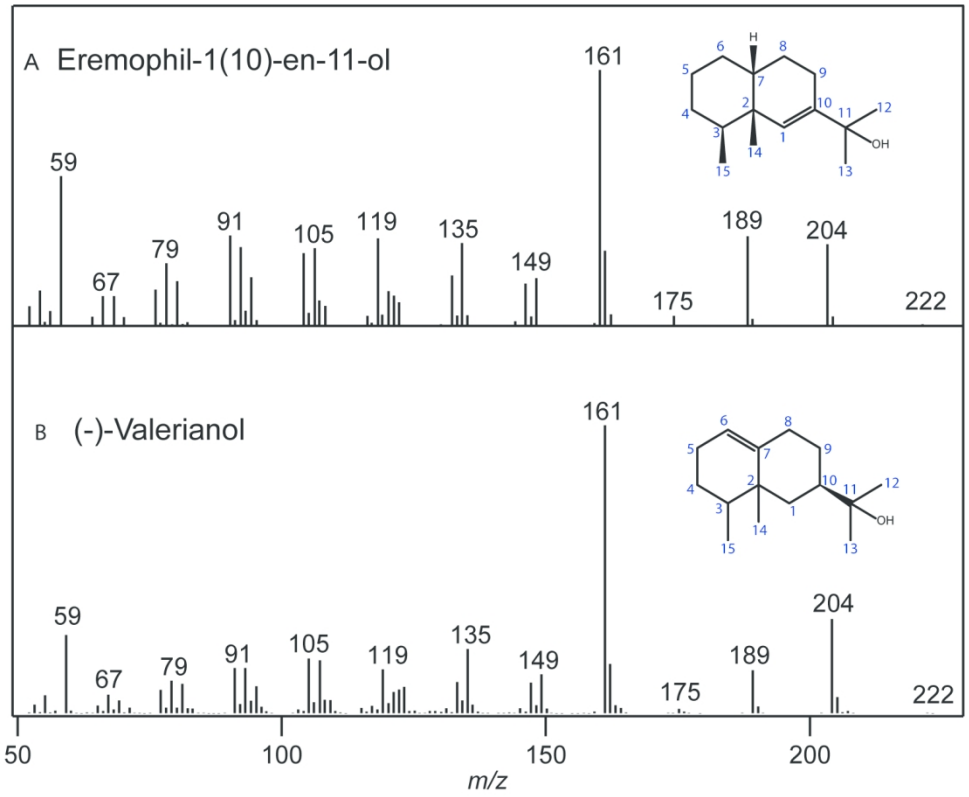

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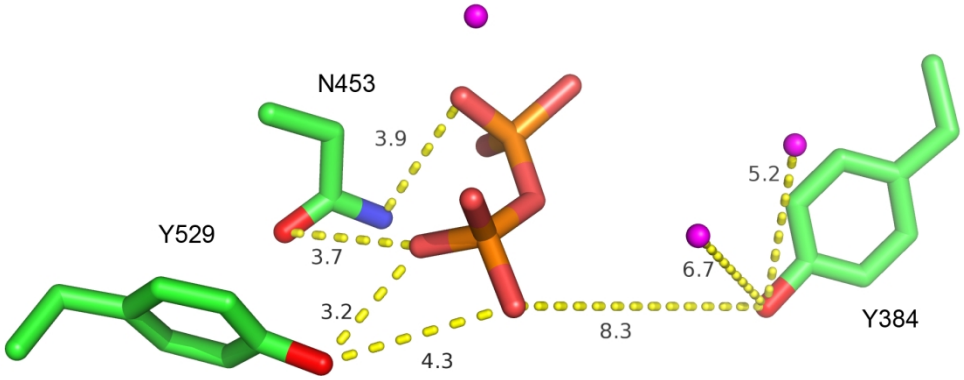

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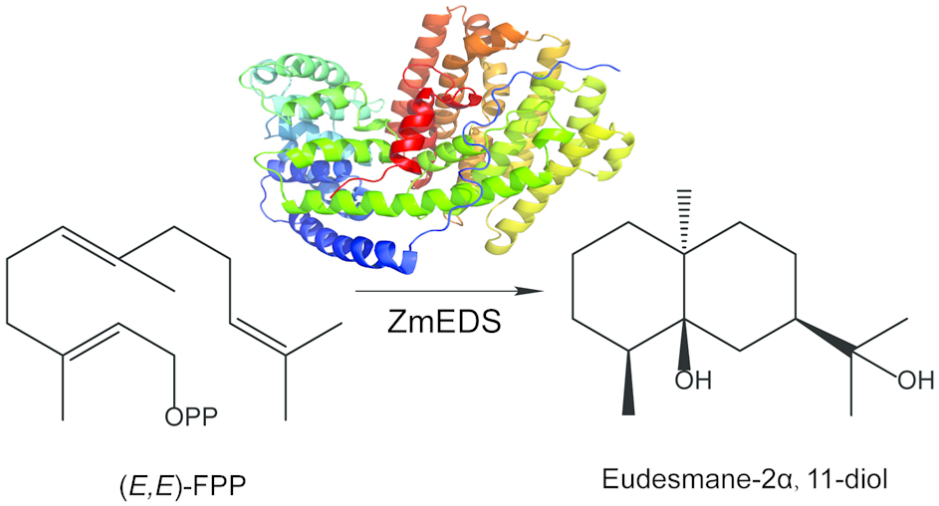

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